

UPCommons

Portal del coneixement obert de la UPC

<http://upcommons.upc.edu/e-prints>

“This is an Accepted Manuscript of an article published by Taylor & Francis in Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering on 05-11-2015, available online:
<http://www.tandfonline.com/doi/full/10.1080/10934529.2015.1079114>”

URL d'aquest document a UPCommons E-prints:
<http://hdl.handle.net/2117/82832>

Article publicat / *Published paper:*

Jové, P., Olivella, M.A., Camarero, S., Caixach, J., Planas, C., Cano, L., De las Heras, F.X. (2016) Fungal biodegradation of anthracene-polluted cork: A comparative study. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, vol 51-1. 70-77. Doi: 10.1080/10934529.2015.1079114

Fungal biodegradation of anthracene-polluted cork - a comparative study

PATRÍCIA JOVÉ¹, MARIA À. OLIVELLA*², SUSANA CAMARERO³, JOSEP CAIXACH⁴, CARLES PLANAS⁴, LAURA CANO¹, FRANCESC X. DE LAS HERAS⁵

¹ Catalan Cork Institute, Palafrugell, Girona, Spain.

² University of Girona, Departament of Chemistry, Girona, Spain.

³ Centro de Investigaciones Biológicas (CSIC), Biotechnology for Lignocellulosic Biomass, Madrid, Spain.

⁴ Department of Environmental Chemistry, IDAEA-CSIC, Mass Spectrometry Laboratory, Barcelona, Spain.

⁵ Universitat Politècnica de Catalunya, Departament of Mining Engineering and Natural Resources. Manresa, Spain.

*Address correspondence to Maria À. Olivella, University of Girona, Departament of Chemistry, Girona, Spain; Phone: +34 972 41 84 16; Fax: +34 972 41 81 50;

E-mail: angels.olivella@udg.edu

Abstract

The efficiency of cork waste in adsorbing aqueous polycyclic aromatic hydrocarbons (PAHs) has been previously reported. Biodegradation of contaminated cork using filamentous fungi could be a good alternative for detoxifying cork to facilitate its final processing. For this purpose, the degradation efficiency of anthracene by three ligninolytic white-rot fungi (*Phanerochaete chrysosporium*, *Irpex lacteus* and *Pleurotus ostreatus*) and three non-ligninolytic fungi which are found in the cork itself (*Aspergillus niger*, *Penicillium simplicissimum* and *Mucor racemosus*) are compared. Anthracene degradation by all fungi was examined in solid-phase cultures after 0, 16, 30 and 61 days. The degradation products of anthracene by *P. simplicissimum* and *I. lacteus* were also identified by GC-MS and a metabolic pathway was proposed for *P. simplicissimum*.

Results shown that all the fungi tested degraded anthracene. After 61 days of incubation approximately 86%, 40% and 38% of the initial concentration of anthracene (i.e. 100 μM) was degraded by *P. simplicissimum*, *P. chrysosporium* and *I. lacteus*, respectively. The rest of the fungi degraded anthracene to a lesser extent (< 30%). As a final remark, the results obtained in this study indicate that *P. simplicissimum*, a non-ligninolytic fungi characteristic of cork itself, could be used as an efficient degrader of PAH-contaminated cork.

Keywords: *Quercus suber* L., bioremediation, biodegradation, anthracene, ligninolytic fungi, *Penicillium simplicissimum*.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic pollutants potentially hazardous to human health because of their carcinogenic, teratogenic and mutagenic character ^[1,2]. Moreover, these hydrophobic compounds persist in the ecosystems due to their low water solubility and its association with organic matter in soils and sediments ^[3,4]. For these reasons, the European Union has established for these compounds very restrictive limits in different kinds of surface waters in the proposal Directive 2006/0129 EC which was approved in 2007/C 97/02 ^[5].

Cork is the bark of the cork oak tree (*Quercus suber* L.), a renewable and biodegradable raw material produced mainly in the Mediterranean region. The main use of cork is for wine bottle stoppers. The cork stoppers industry generates high amounts of by-products of several particle sizes. The annual production of cork waste is around 50,000 tons, which corresponds to an average of 25–30 % of the quantity used in natural cork stopper manufacturing.

Although the most common use of the by-products from the cork industry is combustion for energy production, recent studies have demonstrated their high potential capacity for adsorbing PAH-contaminated water ^[6,7]. After this removal process, the remaining PAH-polluted cork should be treated to facilitate its final processing and to be reused as for example to obtain other cork products like thermal insulation in refrigerators, shoes or packaging ^[8].

Microbiological degradation using various types of organisms is the most frequent process used in the decontamination of surface soil and detoxification hazardous waste. Although in general, PAHs are resistant to most standard bioremediation techniques, they can be oxidized to a certain extend by different bacteria and fungi ^[9,10].

Many biodegradation studies have shown that filamentous fungi are able to metabolize PAHs even more effectively than some bacteria ^[11,12], so that they could be a good alternative for detoxifying PAH-polluted cork. Among fungi, there are several ligninolytic basidiomycetes that have been tested for anthracene degradation: *Bjerkandera* sp., *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes* sp., and *Irpex lacteus* ^[13-17].

Despite several reports having demonstrated the enormous potential of white-rot fungi such as *P. chrysosporium*, *I. lacteus* and *P. ostreatus* in the biodegradation of PAHs, less is known about the degradation capacity of non-ligninolytic filamentous fungi such as those found in cork itself and which could be considered as an excellent alternative for the biodegradation of PAH-contaminated cork.

The biochemical pathway of PAHs by microorganisms has been described by Gray and Thorton ^[18]. Two mechanisms are described to be involved in PAH degradation by fungi: one is based on the action of the intracellular cytochrome P450 monooxygenases, generating epoxides and dihydrodiols intermediates; the other is carried out by extracellular ligninolytic enzymes (i.e. lignin peroxidase, manganese dependent peroxidase and laccase) producing cation radicals from contaminants followed by the formation of quinones ^[19-24]. Due to the fact that the intermediate metabolites generated in PAHs oxidation might be more toxic than the initial compounds, it is important to identify the compounds generated and select the proper degrading microbes.

Exist different microorganisms associated with cork samples during the different manufacturing processes of cork stoppers (i.e. boiled of the planks, cut and punched into cylindrical pieces and cleaned and surface treated with paraffin or/and silicon to obtain the final product). Filamentous fungi such as *A. niger*, *P. simplicissimum* and

Mucor racemosus have been previously found at the final step of the production of cork stoppers^[25] and have been selected in this study because of their ability to remove some xenobiotic compounds^[19, 26-32].

For the cork industry, the development of a new biotechnology based on the use of cork by-products would revalue this residue and would be an incentive for future forest management of this vegetable material. The decontamination of cork adsorbent by using the characteristic fungi of cork itself is an essential step towards understanding its viability and future potential use.

Thus, the aims of this study are (1) to investigate and compare the ability of three ligninolytic fungi (*P. chrysosporium*, *I. lacteus* and *P. ostreatus*) and three non-ligninolytic fungi characteristic of cork itself (*A. niger*, *P. simplicissimum* and *M. racemosus*) to degrade anthracene from contaminated cork samples, (2) to identify the anthracene degradation products generated and (3) to elucidate the degradation pathway in the fungus that shows the highest anthracene degradation.

Material and methods

Chemicals and reagents

Anthracene, 4-terphenyl-d₁₄, anthracene-d₁₀ and phenanthrene-d₁₀ were obtained from Supelco (Bellefonte, USA). Phthalide, dimethyl phthalate, phthalic anhydride and anthrone standards were supplied by Sigma-Aldrich. The ethyl acetate used was super purity grade from Romil (Cambridge). Stock standard solutions of these chemicals were prepared in ethyl acetate and stored in glass bottles in the dark at 4°C. Sodium sulphate was purchased by Panreac and Fluorisil by Sigma-Aldrich.

Organisms and culture conditions

A. niger (CECT 2545), *M. racemosus* (CECT 2670) and *P. simplicissimum* (CECT 20706) belong to the Spanish Collection of type cultures (CECT). *P. ostreatus* (IJFM A579), *P. chrysosporium* (IFKM A547) and *I. lacteus* (IJFM A792) were collected from the Fungal Culture Collection of the Centro de Investigaciones Biológicas (Madrid, Spain).

The fungal strains were grown in 1L erlenmeyer flasks in 250 mL of modified Czapeck-Dox medium prepared with 1% glucose, 0.2% ammonium tartrate and 0.1% yeast extract in shaken conditions (220 rpm) and controlled temperature (28°C). Inocula were prepared by homogenizing the mycelia after 10 days of incubation.

Contaminated cork samples preparation

Samples of cork waste from Catalonia were cut into small pieces (< 10 mm) and milled in a ZM-200 ultra centrifugal mill (Retsch, Netherlands) and the granulometric fraction of 40 to 60 mesh (0.25 to 0.42 mm of particle size) was subsequently used. Cork waste sample selected for this study (CAT_1) has been previously characterized^[33].

Series of 2 g of granulated cork obtained previously was placed inside an erlenmeyer with 50 ml of anthracene solution at 100 µM (0.46 mg g⁻¹). All recipients were mixed with a Vibromatic oscillating shaker at 700 oscillations min⁻¹ during 1 hour. Before inoculation, samples were dried at ambient temperature during 48 hours.

Solid-state fermentation cultures

2 g of cork contaminated with anthracene were inoculated with 6 ml of each homogenized fungal mycellium. The solid-state fermentation (SSF) cultures were

incubated for different periods (0, 16, 30 and 61 days). In addition, a flask with contaminated cork and without fungi was prepared as abiotic control to monitor non-biological losses during all the experiment. All flasks were wrapped with aluminium foil to prevent photodegradation and were maintained under controlled conditions (28°C and 60% humidity) during all time-course assays. Finally the bio-treated cork samples were filtered and frozen until analysis. All the experiments were performed in triplicate.

Anthracene extraction

Defrosted samples were placed in an extraction thimble (Whatman cellulose) and 4-terphenyl d₁₄ (0.1 mg g⁻¹) was added as standard of the extraction. Soxhlet procedure using ethyl acetate for 7 h was performed. After this extraction time, solvent was evaporated to dryness in a rotary evaporator (Aircontrol, Spain) at 40°C. This residue was dissolved in 10 ml of ethyl acetate using an ultrasonic bath (Banderlin Sonorex, Germany) and was subsequently dried over Na₂SO₄ and Fluorisil and evaporated to dryness. The final extract was dissolved in 10 ml of ethyl acetate using ultrasonic bath.

Analysis of anthracene

Anthracene was measured using a gas chromatograph (GC) (Agilent 6890 180 N) coupled to a mass spectrometer (MS) (Agilent 5973N). The separation was achieved using an HP-5MS column (30 m length, 0.25 mm I.D., 0.25 µm film thickness) (J&W Scientific, Folsom, CA, USA). A volume of 1 µl of the sample solution was injected in a splitless mode. GC oven program started at 60°C (1 min), increased by 25°C min⁻¹ to 150 °C, 10°C min⁻¹ to 260°C (held for 20 min) and increased to 270°C (held for 20 min). The carrier gas was helium (99.999 %) from Abello Linde (Barcelona, Spain) with a constant flow rate of 1 mL min⁻¹. Internal standard calibration using phenanthrene d₁₀

was used for anthracene quantification. The mass spectrometer was operated in SIM mode detecting the following ion masses: 178, 188 and 244. All analysis were performed in triplicate.

Analysis of anthracene degradation products

Anthracene degradation products were analyzed by GC coupled to MS on an integrated quadrupole MD-800 (Thermo, Manchester UK). The GC separation was performed on a DB-5 column (60 m length, 0.25 mm I.D., 0.25 μm film thickness) from J&W (Folsom, CA, USA). Splitless injection mode was used for 1 min and injection volume was 1 μL . Oven temperature program was from 70°C held 1 min to 310°C maintained 1 min at 7°C min^{-1} . Injector and interphase temperature were 270°C and 280°C, respectively. Helium was used as carrier gas at a constant head pressure of 100 kPa. Mass spectrometry was performed using the electron ionization mode (EI+) at 70 eV of ionization energy. Ion source temperature was 250°C. Acquisition was carried out in the full scan mode from m/z 45 to 450 at 0.5 s scan^{-1} with an interscan time of 0.1 s. Analytes were quantified from their response factor related to anthracene- d_{10} , used as internal standard. Blanks with hexane and without sample were also analyzed to assess the possible contamination of samples by phthalates from plastic during preparation, storage and analysis. All analysis were performed in duplicate.

Identification of anthracene degradation products were confirmed using standards of metabolites (2-anthrone, 9,10 anthraquinone, phthalide acid, and phthalide) as shown in Fig.1.

Results and discussion

Anthracene biodegradation

The percentages of anthracene removal from contaminated cork after 16, 30 and 61 days of solid-state fermentation (SSF) with the different fungi are shown in Fig.2. To calculate the biodegradation percentages, SSF samples of 0 day-incubation period were used as reference for the initial PAH concentration. The abiotic control showed anthracene losses less than 1% (data not shown).

As shown in Fig.2, all fungi were able to remove anthracene from cork after 61 days of incubation, although with rather different yields. *P. simplicissimum* showed the highest percentage of anthracene degradation (86%). In contrast, *P. ostreatus* demonstrated the lowest percentage of anthracene degradation (15%). The final percentages of anthracene removal obtained with the other white-rot fungi, *P. chrysosporium* and *I. lacteus*, were higher (~40%) than those obtained with the non-ligninolytic fungi *A. niger* (31%) or *M. racemosus* (24%).

Fig.3 shows the degradation of the anthracene peak by *P. simplicissimum* over time. It was calculated that 42% of anthracene degradation was produced during the first sixteen days.

These results are consistent with the fungal growth observed on the contaminated cork. *P. ostreatus* showed the poorest colonization of the substrate compared to the control (without fungi). In contrast, *P. simplicissimum* grew rapidly and the mycelium covered the whole cork surface after 16 days of incubation (Fig. 4). The rest of the fungi exhibited moderate growth.

In general, anthracene removal was observed to increase along with the increase in time period of solid-state fermentation (SSF), except in the cases of the degradation percentages attained with *A. niger* and *P. ostreatus* which remained fairly constant after the first 16 days of incubation (Fig. 2).

Table 1 summarizes the data reviewed from literature on anthracene degradation yields produced by filamentous and non-filamentous fungi. Although it is difficult to compare because of the different parameters used some general aspects are worth mentioning. The degradation values ranged from 20% up to the complete removal of anthracene. Moreover, a great variability in the percentages of anthracene degradation within each ligninolytic fungi was observed. These variations depended on various factors such as the initial concentration of the target pollutant or the culture conditions such as type of medium, pH or temperature and the bioavailability of the pollutant. It was also observed that the percentage of PAH biodegradation was significantly higher in liquid than in solid culture conditions, probably due to the decreased PAH availability in solid medium^[34,35]. Even so, there are some studies on solid cultures that demonstrate higher degradation rates (Table 1), probably due to the effect of the different factors mentioned above^[14, 17, 36].

With ligninolytic fungi, similar percentages of anthracene degradation were obtained by *I. lacteus* after 120 days of incubation in creosote contaminated soil with a 32-38% PAH mix^[15], and in sterilized soil after 70 days^[17]. On the contrary, PAH degradation studies with *P. chrysosporium* and *P. ostreatus* in a solid medium, reported percentages of anthracene removal significantly higher (over 90%)^[14, 36], to those obtained in this study (around 40 and 15%, respectively). Note that, the high percentages of anthracene removal reported in the literature for *P. chrysosporium* were performed at higher initial

anthracene concentrations (0.05-1 g L⁻¹) compared to that used in this study (0.017 g L⁻¹) [13, 14, 37]. Bhatt et al. [38] and Byss et al. [15] described the effect of the initial PAH concentration on anthracene biodegradation. However, while Bhatt et al. [38] had reported increments of 20-30% in anthracene degradation when the initial concentration of the PAH was duplicated, Byss et al. [15] did not find any significant differences in the PAH removal by *P. ostreatus* and *I. lacteus* when the initial concentration was increased from 0.7 mg kg⁻¹ to 32 mg kg⁻¹.

It is worth noting the high anthracene degradation efficiency found in this study for *P. simplicissimum* (86%), and which were similar to the above mentioned values found in the literature for the three selected ligninolytic fungi (Table 1). Similar anthracene degradation values (around 30 %) to those obtained in this study (29%) have been reported for *Aspergillus niger* [39]. However, to the best of our knowledge, this study is the first in which anthracene degradation for the non-ligninolytic fungi *Penicillium simplicissimum* and *Mucor racemosus* is described.

The degradation studies with high molecular weight PAHs for non-ligninolytic fungi revealed that Zygomycetes, and in particular *M. racemosus*, appeared as one of the most efficient taxonomic groups to degrade these types of PAHs (4-7 rings) such as pyrene and benzo[a]pyrene [11,40]. *P. simplicissimum* and *M. racemosus* have also demonstrated the ability to remove pyrene in a liquid synthetic medium after 2 days (2.4 and 3.26 mg g⁻¹ of pyrene, respectively) [19,30]. Since the above-mentioned PAHs are more recalcitrant to biodegradation than low molecular PAHs, the capability of the non-ligninolytic fungi *P. simplicissimum*, *M. racemosus* and *A. niger* to remove, for instance anthracene, could be inferred; as was demonstrated in this study under SSF conditions.

Given that *P. simplicissimum* is a fungus characteristic of cork ^[41], the outstanding capability of this fungi to remove anthracene from contaminated cork, demonstrated here, represents a valuable step forward for the future development of a bio-technique to detoxify contaminated cork. Therefore, the intermediate oxidation products were analyzed to assess the degradation pathway used by *P. simplicissimum* and whether the toxic compound was transformed onto harmless substances or not. For the sake of comparison, the intermediates generated during the oxidation of anthracene with the ligninolytic fungi *I. lacteus* were also identified.

Metabolic products from the biodegradation of anthracene by P. simplicissimum and I. lacteus

Following the biotreatment of contaminated cork with *P. simplicissimum* and *I. lacteus*, after 0, 16 and 61 days of incubation, the anthracene degradation products obtained were identified using GC-MS. The retention times, mass spectral characteristics of anthracene and its metabolites analyzed in all cork extracts are shown in Table 2.

Anthraquinone, anthrone and traces of phthalic acid and phthalic anhydride were found in the final extracts of *P. simplicissimum* and *I. lacteus*. In contrast, compounds such as 9-anthrol, 2-(2'-hydroxybenzoyl)-benzoic acid and anthracene trans-1,2-dihydrodiol were not found in any extract from the respective periods.

While the maximum abundance of anthrone, which was 0.16% and 0.15% of the initial concentration of anthracene for *I. lacteus* and *P. simplicissimum* cultures, respectively, was detected after 16 days, it was not detected at the end of the SSF (61 days) with either fungus. 9-10-anthraquinone was found to be the principal oxidation product detected in the 16-day samples. It accumulated in the both fungal cultures after 16 days

(8.5% and 1.2% for *I. lacteus* and *P. simplicissimum*, respectively) and decreased after 61 days (5.3% and 0.07% for *I. lacteus* and *P. simplicissimum*, respectively). This suggests that, although 9-10-anthraquinone is a stable intermediate ^[42], it is not the end oxidation product ^[43]. This was confirmed by the detection of traces of phthalic acid and phthalic anhydride (0.58% and 0.09% for *I. lacteus* and *P. simplicissimum*, respectively) after 16 days of SSF. These results demonstrate the fungal capability to perform further degradation steps until cleavage of the aromatic ring.

In line with these intermediate products detected, Fig.5 shows the proposed pathway for anthracene degradation by *P. simplicissimum*. This degradation pathway has previously been reported for ligninolytic fungi ^[11,42,44-47] and non-ligninolytic fungi ^[48]. Accumulation of PAH-quinones, is characteristic of PAH degradation by white-rot fungi under ligninolytic conditions ^[11,13,49,50], and constitutes a good detoxification alternative as quinones are less toxic ^[51,52] and more bioavailable than the initial PAH and subsequently they may be readily degraded by bacteria ^[53]. In this study, production of 9,10-anthraquinone during the degradation of anthracene by *P. simplicissimum* (although in a significantly lower amount than with *I. lacteus*) suggests the involvement of some ligninolytic oxidoreductase activity (laccase or peroxidase) in this process. According to GenBank and UniProt databases, laccase and peroxidase activity has been also described in other *Penicillium* sp. such as *P. roqueforti*, *P. digitatum*, *P. oxalicum*, *P. chrysogenum*, *P. citrinum*, *P. expansum* and only laccase activity has been detected in *P. marnefroi* and *P. stiptatatus*

The presence of anthrone in the final extract of non-ligninolytic fungi evidences the possible involvement of the extracellular ligninolytic system in anthracene degradation by *Penicillium simplicissimum* ^[13,54]. Clemente et al. ^[55] and Zeng et al. ^[31] also detected

ligninolytic activity during the growth of non-ligninolytic fungi in PAH. In the case of *P. simplicissimum*, ligninolytic activity has been shown to mainly occur during the primary metabolism^[56]. The presence of ligninolytic enzymes in non-ligninolytic fungi such as *Trichoderma reesei* and *Fusarium proliferation* has also been elucidated^[57,58].

Conclusions

Results obtained in this work have shown that, to a greater or lesser degree, white-rot fungi (i.e. *Phanerochaete chrysosporium*, *Irpex lacteus* and *Pleurotus ostreatus*) and ascomycete fungi characteristics of cork (i.e. *Aspergillus niger*, *Penicillium simplicissimum* and *Mucor racemosus*) are able to degrade anthracene in anthracene-polluted cork. After 61 days of incubation, *P. simplicissimum* produced the highest degree of anthracene removal (86%) followed by *P. chrysosporium*, *I. lacteus* (40%), *A. niger* (31%), *M. racemosus* (24%) and *P. ostreatus* (15%).

Anthraquinone, anthrone and traces of phthalic acid and phthalic anhydride were detected as intermediate products from anthracene degradation by *P. simplicissimum* and *I. lacteus*. According to this, the following degradation pathway for *P. simplicissimum* was proposed: initially, anthracene was oxidized to anthrone, which was then further transformed to anthraquinone, and finally, trace levels of phthalic acid were produced by ring cleavage. The proposed pathway for anthracene degradation by *P. simplicissimum* is similar to that previously described for ligninolytic fungi. This fact is beneficial for the main purpose of this study, i.e. the detoxification of PAH-contaminated cork, because anthraquinone is less contaminating and more easily biodegradable than anthracene

Thus, the biodegradation capacity and detoxification pathway obtained from anthracene, suggest that *P. simplicissimum* could be used efficiently to bio-remediate PAHs polluted cork.

Acknowledgements

This research was funded by the Spanish Ministerio de Ciencia e Innovación as part of the project CTM 2009-07162 and CTM2010-15185. Thanks to the Cork Center Laboratory to its technical support and AECORK for providing the cork samples.

References

- [1] International Agency for Research on Cancer (IARC). Monographs on the evaluation of carcinogenic risks to humans. Lyon, France; **1991**.
- [2] Leonardi, V.; Giubilei, M.A.; Federici, E.; Spaccapelo, R.; Šašek, V.; Novotný, C.; Petroccioli, M.; D'Annibale, A. Mobilizing agents enhance fungal degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria soil. *Biotechnol. Bioeng.* **2008**, *101*, 273-285.
- [3] Fernández, P.; Grifoll, A.M.; Solanas, A.M.; Bayona, J.M.; Albaigés, J. Bioassay-directed chemical analysis of genotoxic components in coastal sediments. *Environ. Sci. Technol.* **1992**, *26*, 817-829.
- [4] Wild, S.R.; Jones, K.C. Polynuclear aromatic hydrocarbons in the United Kingdom environment: a preliminary source inventory and budget. *Environ. Pollut.* **1995**, *88*, 91-108.
- [5] 2007/C 97/02 Opinion of the European Economic and Social Committee on the Proposal for a Directive of the European Parliament and of the Council on environmental quality standards in the field of water policy and amending Directive 2000/60/EC, *Off. J. Eur. Union* C97; **2007**.
- [6] Olivella, M.À.; Jové, P.; Oliveras, A. The use of cork waste as a biosorbent for persistent organic pollutants – Study of adsorption/desorption of polycyclic aromatic hydrocarbons. *J. Environ. Sci. Health Part A* **2011**^a, *46*, 824–832.

- [7] Olivella, M.À.; Jové, P.; Şen, A.; Pereira, H.; Villaescusa, I.; Fiol, N. Sorption performance of *Quercus cerris* cork with polycyclic aromatic hydrocarbons and toxicity testing. *Bioresources* **2011**^b, *6*, 3363-3375.
- [8] Gil, L. Cork composites: a review. *Materials* **2009**, *2*, 776-789.
- [9] Eggen, T.; Sasek, V. Use of edible and medicinal oyster mushroom [*Pleurotus ostreatus* (Jacq.: Fr.) Kumm] spent compost in remediation of chemically polluted soils. *Int. J. Medicinal Mushrooms* **2002**, *4*, 255-261.
- [10] Haristash, A.K.; Kaushik, C.P. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. *J. Hazard. Mater.* **2009**, *169*, 1-15.
- [11] Cerniglia, C.E. Fungal metabolism of PAHs. *J. Ind. Microbiol. Biotechnol.* **1997**, *19*, 324-333.
- [12] Peng, R.H.; Xiong, A.S.; Xue, Y.; Fu, X.Y.; Gao, F.; Zhao, W.; Tian, Y.S.; Yao, Q.H. Microbial biodegradation of polycyclic aromatic hydrocarbons – review. *FEMS Microbiol. Rev.* **2008**, *32*, 927-955.
- [13] Field, A.; DeJong, E.; Costa, G.F.; DeBont, J.A.M. Biodegradation of polycyclic aromatic hydrocarbons by new isolates for white rot fungi. *Appl. Environ. Microbiol.* **1992**, *58*, 2219–2226.
- [14] Bishnoi, K.; Kumar, R.; Bishnoi, N.R. Biodegradation of polycyclic aromatic hydrocarbons by white rot fungi *Phanerochaete chrysosporium* in sterile and unsterile soil. *J. Sci. Ind. Res.* **2008**, *67*, 538-542.
- [15] Byss, M.; Elhottová, D.; Třiska, J.; Baldrian, P. Fungal bioremediation of the creosote-contaminated soil: Influence of *Pleurotus ostreatus* and *Irpex lacteus* on

polycyclic aromatic hydrocarbons removal and soil microbial community composition in the laboratory-scale study. *Chemosphere* **2008**, *73*, 1518-1523.

[16] Novotný, Č.; Cajthaml, T.; Svobodová, K.; Šušla, M.; Šašek, V. *Irpex lacteus*, a white-rot fungus with biotechnological potential – review. *Folia Microbiol.* **2009**, *54*, 375-390.

[17] Borràs, E.; Caminal, G.; Sarrà, M.; Novotný, Č. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil. *Soil Biol. Biochem.* **2010**, *42*, 2087-2093.

[18] Gray, P.H.; Thorton, H.G. Soil bacteria that decompose certain aromatic compounds. *Zentr. Bakt. Parasitenk.* **1928**, *73*, 74-96.

[19] da Silva, M.; Umbuzeiro, G.A.; Pfenning, L.H.; Canhos, V.P.; Esposito, E. Filamentous fungi isolated from estuarine sediments contaminated with industrial discharges. *Soil Sediment. Contam.* **2003**, *12*, 345-356.

[20] Tortellá, G.R.; Diez, M.D. Fungal diversity and use in decomposition of environmental pollutants. *Crit. Rev. Microbiol.* **2005**, *31*, 197-212.

[21] Cajthaml, T.; Erbanová, P.; Kollmann, A.; Novotný, Č.; Šašek, V.; Mougin, C. Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus*. *Folia Microbiol.* **2008**, *53*, 289-294.

[22] Wu, Y.; Teng, Y.; Li, Z.; Liao, X.; Luo, Y. Potential role of polycyclic aromatic hydrocarbons (PAHs) oxidation by fungal laccase in the remediation of an aged contaminated soil. *Soil Biol. Biochem.* **2008**, *40*, 789-796.

- [23] Čvančarová, M.; Křesinová, Z.; Filipová, A.; Covino, S.; Cajthaml, T. Biodegradation of PCBs by ligninolytic fungi and characterization of the degradation products. *Chemosphere* **2012**, *88*, 1317-1323.
- [24] Pozdnyakova, N.N. Involvement of the ligninolytic system of white-rot and litter-decomposing fungi in the degradation of polycyclic aromatic hydrocarbons. Review article. *Biotechnol. Res. Int.* **2012**, 243-267.
- [25] Centeno, S. Evaluación de la calidad microbiológica de tapones de corcho para vinos elaborados en Catalunya. Tesis doctoral. Universidad Autónoma de Barcelona. Facultad de Ciencias. Departamento de genética y microbiología, 2001.
- [26] Wang, X.; Yu, X.; Bartha, R. Effect of bioremediation on polycyclic aromatic hydrocarbon residues in soil. *Environ. Sci. Technol.* **1990**, *24*, 1086–1089.
- [27] Sack, U.; Heinze, T.M.; Deck, J.; Cerniglia, C.E.; Cazau, M.C.; Fritsche, W. Novel metabolites in phenanthrene and pyrene transformation by *Aspergillus niger*. *Appl. Environ. Microbiol.* **1997**, *63*, 2906-2909.
- [28] Yogambal, R.K.; Karegoudar, T.B. Metabolism of polycyclic aromatic hydrocarbons by *Aspergillus niger*. *Indian J. Exp. Biol.* **1997**, *35*, 1021-1030.
- [29] Krivobok, S.; Miriouchkine, E.; Seigle-Murandi, F.; Benoit-Guyod, J.L. Biodegradation of anthracene by soil fungi. *Chemosphere* **1998**, *37*, 523-530.
- [30] Ravelet, C.; Grosset, C.; Montuelle, B.; Benoit-Guyod, J.L.; Alary, J. Liquid chromatography study of pyrene degradation by two micromycetes in a freshwater sediment. *Chemosphere* **2011**, *44*, 1541-1546.

- [31] Zeng, G.M.; Yu, H.Y.; Huang, H.L.; Huang, D.L.; Chen, Y.N.; Huang, G.H. Laccase activities of a soil fungus *Penicillium simplicissimum* in relation to lignin degradation. *World J. Microbiol. Biotechnol.* **2006**, *22*, 317-324.
- [32] Okoro, C.C. Biodegradation of hydrocarbons in untreated produce water using pure fungal cultures. *Afr. J. Microbiol. Res.* **2008**, *2*, 217-223.
- [33] Jové, P.; Olivella, M.À.; Cano, L. Study of the variability in chemical composition of bark layers of *Quercus suber* L. from different production areas. *Bioresources* **2011**, *6*, 1806-1815.
- [34] Boyle, D.; Wiesner, C.; Richardson, A. Factors affecting the degradation of polycyclic aromatic hydrocarbons in soil by white rot fungi. *Soil Biol. Biochem.* **1998**, *30*, 873-882.
- [35] Yuan, S.Y.; Shiung, L.C.; Chang, B.V. Biodegradation of polycyclic aromatic hydrocarbons by inoculated microorganisms in soil. *Bull. Environ. Contam. Toxicol.* **2002**, *69*, 66-73.
- [36] Okparanma, R.N.; Ayotamuno, J.M.; Davis, D.D.; Allagoa, M. Mycoremediation of polycyclic aromatic hydrocarbons (PAH)-contaminated oil-based drill-cuttings. *Afr. J. Biotechnol.* **2011**, *10*, 5149-5156.
- [37] Bumpus, J.A. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **1989**, *55*, 154-158.
- [38] Bhatt, M.; Cajthaml, T.; Šašek, V. Mycoremediation of PAH-contaminated soil. *Folia Microbiol.* **2002**, *47*, 255-258.

- [39] Giraud, F.; Guiraud, P.; Kadri, M.; Blake, G.; Steiman, R. Biodegradation of anthracene and fluoranthene by fungi isolated from an experimental constructed wetland for wastewater treatment. *Wat. Res.* **2001**, *35*, 4126-4136.
- [40] Passarini, M.R.Z.; Sette, L.D.; Rodrigues, V.N. Improved extraction method to evaluate the degradation of selected PAHs by marine fungi grown in fermentative medium. *J. Braz. Chem. Soc.* **2011**, *22*, 564-570.
- [41] Pi, M. Hongos y micotoxinas en tapones de corcho. Propuesta de limites micológicos aceptables.
<http://www.tdx.cat/bitstream/handle/10803/3901/mpc1de1.pdf?sequence=1> (Accessed November 2014).
- [42] Cajthaml, T.; Möder, M.; Kačer, P.; Šašek, V.; Popp, P. Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. *J. Chromatogr. A* **2002**, *974*, 213-222.
- [43] Cañas, A.I.; Camarero, S. Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. *Biotechnol. Adv.* **2007**, *28*, 694-705.
- [44] Bezalel, L.; Hadar, Y.; Fu, P.P.; Freeman, J.P.; Cerniglia, C.E. (1996). Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **1996**, *62*, 2547-2553.
- [45] Baborová, P.; Möder, M.; Baldrian, P.; Cajthamlová, K.; Cajthaml, T. Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus*, and degradation of polycyclic aromatic hydrocarbons by the enzyme. *Res. Microbiol.* **2006**, *157*, 248-253.

- [46] Eibes, G.; Cajthaml, T.; Moreira, M.T.; Feijoo, G.; Lema, J.M. Enzymatic degradation of anhracene, dibenzothiophene and pyrene by manganese peroxidasa in media containing acetone. *Chemosphere* **2006**, *64*, 408-414.
- [47] Chigu, N.L.; Hirose, S.; Nakamura, C.; Teramoto, H.; Ichinose, H.; Wariishi, H. Cytochrome P450 monooxygenases involved in anthracene metabolism by the White-rot basidiomycete *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1907-1916.
- [48] Ye, J.S.; Yin, H.; Qiang, J.; Peng, H.; Qin, H.M. Biodegradation of anthracene by *Aspergillus fumigatus*. *J. Hazard. Mater.* **2011**, *185*, 174-181.
- [49] Novotný, Č.; Erbanová, P.; Šašek, V.; Kubátová, A.; Cajthaml, T.; Lang, E.; Krahl, J.; Zadrazil, F. Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation* **1999**, *10*, 159-168.
- [50] Schützendübel, A.; Majcherczyk, A.; Johannes, C.; Hüttermann, A. Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. *Int. Biodeterior. Biodegrad.* **1999**, *43*, 93-100.
- [51] Bondy, G.S.; Armstrong, C.L.; Dawson, B.A.; Héroux-Metcalf, C.; Neville, G.A.; Rogers, C.G. Toxicity of structurally related anthraquinones and anthrones to mammalian cells in vitro. *Toxicol. in Vitro* **1994**, *8*, 329-335.
- [52] Gómez-Alvarez, M.; Poznyak, T.; Ríos-Leal, E.; Silva-Sánchez, C. Anthracene decomposition in soils by conventional ozonation. *J. Environ. Manage.* **2012**, *113*, 545-551.

- [53] Mueller, E.J.; Loida, P.J.; Sligar, S.G. Twenty-five years of P450 cam Research. Cytochrome P450: Structure, Mechanism and Biochemistry. Plenum Press, New York, **1995**.
- [54] Haemmerli, S.D. Degradation of polycyclic aromatic hydrocarbons. In Lignin peroxidase and the lignolytic system of *Phanerochaete chrysosporium*. Ph.D. Dissertation, Swiss Federal Institute of Technology, Zurich, Switzerland, **1988**.
- [55] Clemente, A.R.; Anazawa, T.A.; Durrant, L.R. Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. Braz. J. Microbiol. **2001**, *32*, 255-261.
- [56] Yu, H.Y.; Zeng, G.M.; Huang, G.H.; Huang, D.L.; Chen, Y.N. (2005). Lignin degradation by *Penicillium simplicissimum*. Huan Jing Ke. Xue. **2005**, *26*, 167-171.
- [57] Kwon, S.I.; Anderson, A.J. Genes for multicopper proteins and laccase activity: common features in plant-associated *Fusarium* isolates. Can. J. Bot. **2002**, *80*, 536-570.
- [58] Levasseur, A.; Saloheimo, M.; Navarro, D.; Andberg, M.; Pontarotti, P.; Kruus, K. (2010). Exploring laccase-like multicopper oxidase genes from the ascomycete *Trichoderma reesei*: a functional, phylogenetic and evolutionary study. BMC Biochem. **2010**, *11*, 1471-2091.

FIGURE CAPTIONS

Figure 1.- Chromatogram of standards of the metabolites analyzed from anthracene degradation (1-anthracene, 2-anthrone, 3- 9,10 anthraquinone, 4-phthalide acid, and 5-phthalide).

Figure 2. Removal of anthracene from contaminated cork by solid-state fermentation with three non-ligninolytic fungi (Ps: *Penicillium simplicissimum*; An: *Aspergillus niger*; Mr: *Mucor racemosus*) and three ligninolytic fungi (Il: *Irpex lacteus*; Pc: *Phanerochaete chrysosporium*; Po: *Pleurotus ostreatus*) after 16, 30 and 61 days of incubation. Bars are standard deviations of triplicate analysis.

Figure 3. Chromatogram of the peak of anthracene degradation by *P. simplicissimum* after 0 (black), 16 (red) and 61 (green) days of incubation.

Figure 4. Abiotic control (i.e. without fungus) (C) and fungal growth (Po: *Pleurotus ostreatus*; Ps: *Penicillium simplicissimum*) in contaminated cork after 61 days of incubation.

Figure 5.- Proposed pathway of anthracene degradation by *Penicillium simplicissimum*.

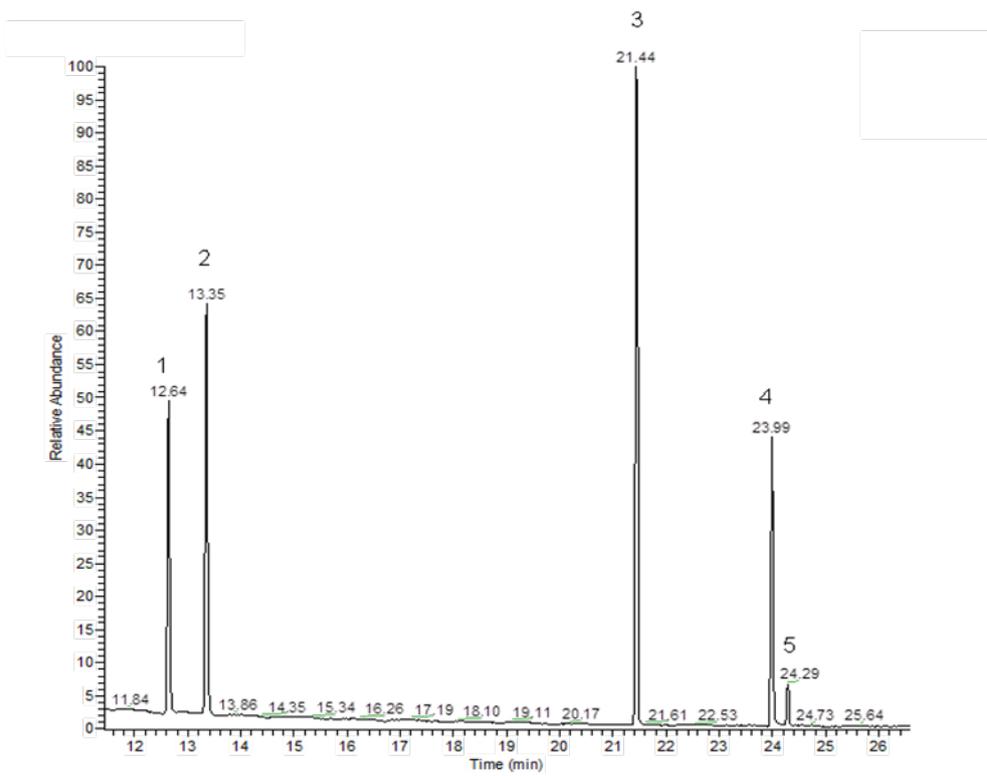


Fig. 1

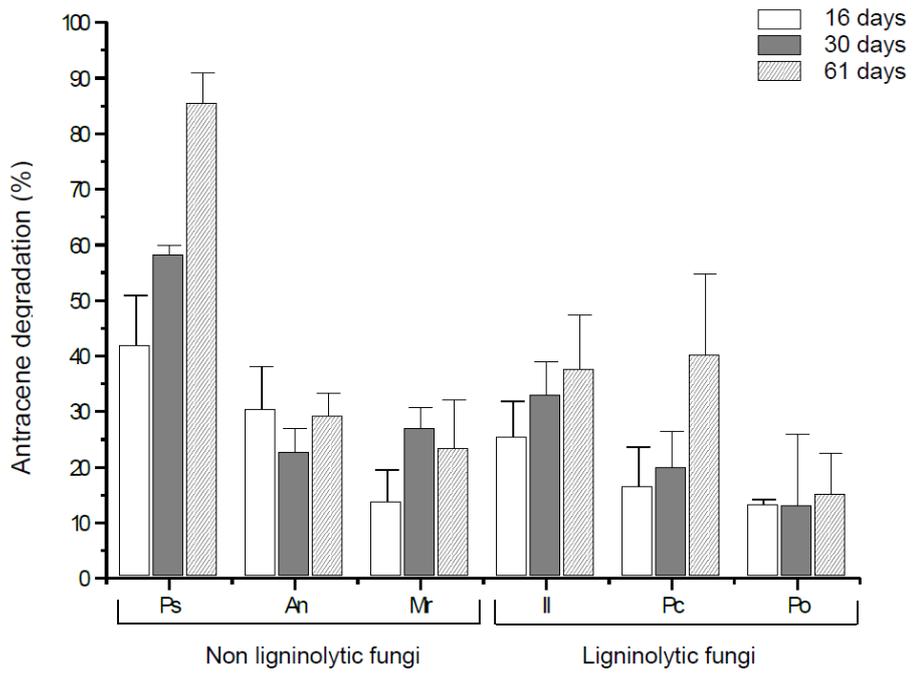


Fig. 2

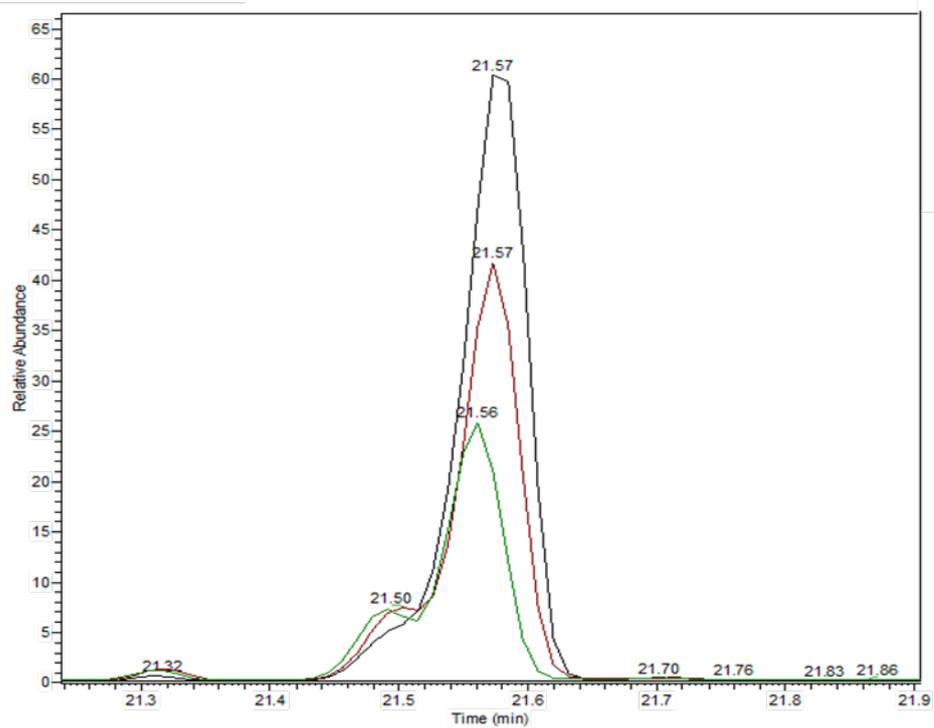


Fig. 3



Fig. 4

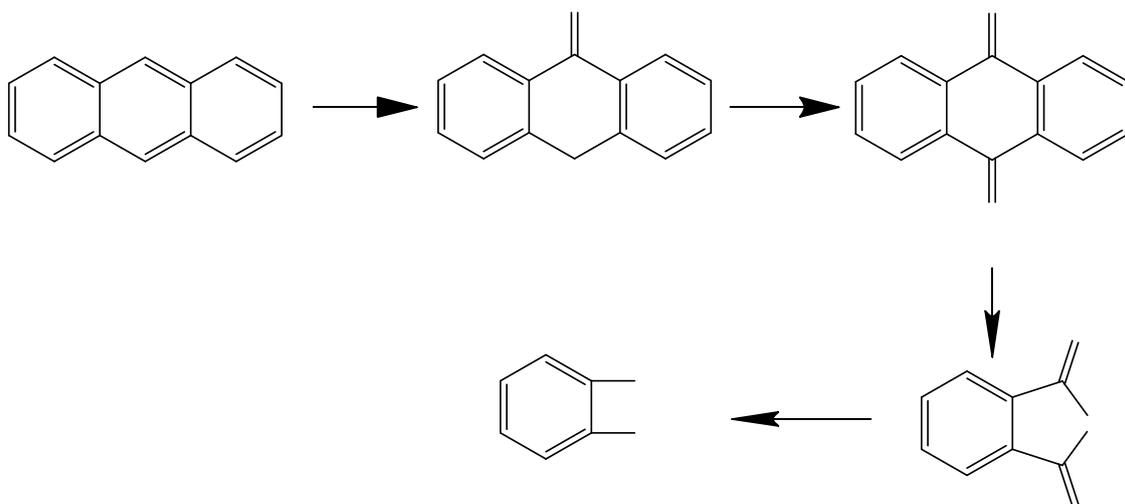


Fig. 5

Table 1. Comparison of anthracene degradation by several fungi reported in the literature.

Organism	Growth substract	Initial anthracene concentration	pH	T (°C)	Incubation time (days)	% removal	Reference
<i>Irpex lacteus</i>	Contaminated cork	0.017 g/L	-	28	61	37.8	This study
	Liquid culture	0.025 g/L	-	28	14	60	Novotný et al. [16]
	Tyndallized dry soil	0.05 g/L	-	26	90	49	Novotny et al. [16]
	Liquid culture medium	0.025 g/L	-	-	50	>95	Cajhaml et al. [42]
	Liquid culture medium	0.025 g/L	-	-	50	77.7-99.2	Cajhaml et al. [21]
	Polluted soil	0.7 mg/Kg	-	15	120	32	Byss et al. [15]
		32 mg/Kg	-	15	120	38	
	Sterilized soil	18.8 mg/Kg	-	-	35	60.6	Borràs et al. [17]
		14.2 mg/Kg			70	42.9	
	Unsterilized soil	18.8 mg/Kg	-	-	35	47.3	Borràs et al. [17]
		14.2 mg/Kg			70	47.8	
Industrial soil	229 mg/kg	6.49	26	98	72.9	Bhatt et al. [38]	
	99 mg/kg	6.85	26	98	47.8		
Sterilized quartz sand	1.6 g/L	-	28	42	69.4	Leonardi et al. [2]	
<i>Phanerochaete chrysosporium</i>	Contaminated cork	0.017 g/L	-	28	61	40.2	This study
	Liquid culture medium with anthracene oil	0.05 g/L	-	39	27	98	Bumpus [37]
	Liquid culture medium	1 g/L	-	-	28	99.2	Field et al. [13]
	Liquid culture medium	0.01 g/L	-	37	4	58	Krivobok et al. [29]
	Sterilized soil	5 mg/Kg	7	30	42	92.6	Bishnoi et al. [14]
<i>Pleurotus ostreatus</i>	Contaminated cork	0.017 g/L	-	28	61	15.2	This study
	Creosote contaminated soil	0.7 mg/Kg	-	15	120	64	Byss et al. [15]
		32 mg/Kg				77	
	Liquid culture medium	0.5 g/L	-	22	12	62	Schützendübet et al. [50]
	Liquid culture medium	8.3 g/L	-	28	21	74	Bezalel et al. [44]
	Polluted soil	220 mg/kg	-	-	84	50	Eggen and Sasek [9]
	Sterilized quartz sand	1.6 g/L	-	28	42	78	Leonardi et al. [2]
	Industrial soil	229 mg/kg	6.49	26	98	70.3	Bhatt, et. al. [38]
99 mg/kg		6.85	26	98	32.6		
Drill cuttings	10.92 mg/Kg	-	30	56	100	Okparanma et al. [36]	
<i>Aspergillus niger</i>	Contaminated cork	0.017 g/L	-	28	61	29.4	This study
	Liquid culture medium	0.01 g/L	-	22-24	4	27.1-37.5	Giraud et al. [39]
<i>Penicillium simplicissimum</i>	Contaminated cork	0.017 g/L	-	28	61	85.5	This study
<i>Mucor racemosus</i>	Contaminated cork	0.017 g/L	-	28	61	23.5	This study

Table 2. - Retention data and mass spectral characteristics of anthracene and its metabolites analyzed in all cork extracts.

Product no.	t _R (min)	m/z ions (relative intensity)	Structural suggestion
1	21.44	178 (100), 179 (15.7), 176 (14.1), 89 (7.6)	anthracene
2	23.99	194 (100), 165 (98.4), 138 (49.6), 81 (37.1)	Anthrone
3	24.29	208 (100), 180 (64.2), 152 (58.8), 126 (4.4), 76 (5.9)	9,10-anthraquinone
4 and 5	12.64	104 (100), 148 (18.7)	Phthalic acid and phthalic anhydride
6	-*	166 (100), 194 (38.0)	Anthracene trans-1,2-dihydrodiol
7	-*	224 (100), 196 (41.0), 168 (32.8), 242 (14.4), 139 (14.1)	2-(2'-Hydroxybenzoyl)-benzoic acid
8	13.35	105 (100), 77 (40.9), 134 (12.7), 51 (9.0)	phthalide

* Analytes identified by m/z ions and relative intensities

